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PROLIFERATION RATE AND CELL SIZE ANALYSES OF HUMAN PERIPHERAL BLOOD SUSPENSION STEM CELLS FROM THREE CULTURING TERMS POPULATIONS

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ABSTRACT

Stem cells that made up of embryonic stem cells and adult stem cells are well known for their ability to self renew and differentiate into matured cells. Adult stem cells isolated from peripheral blood system were used as the study sample. The objective of this study was to determine the relationship between size and proliferation potential of stem cells from 3 different culturing terms, i.e., short (7 days), medium (14 days), and long (30 days) terms. Density gradient centrifugation was applied in isolating mononucleated cells (MNCs) from peripheral blood sample. Isolated MNCs were washed with Hank's Balanced Salt Solution and Phosphate Buffer Saline. The MNCs which cultured in complete media were then subjected to proliferation analysis every day for a time period of 30 days. RT-PCR analyses and cell size measurements were carried out at day 7, 14, and 30. Activation of *KIT* and *SLAMF1* marker genes during RT-PCR analyses indicated that the isolated MNCs were stem cells. Analyses of proliferation rate and cell size showed that short term stem cells have the largest cell size with the lowest proliferation potential. While, medium term stem cells gave rise to a smaller stem cell population with higher proliferation potential compared to short term stem cells. Long term stem cells have smallest cell size with highest proliferation potential. In conclusion, each culturing term cell population had their own sizes and can be isolated based on those sizes.

Key words: Stem cells, Proliferation Potential, RT-PCR

INTRODUCTION

Higher immunocompatibility with lower Graft-versus-host disease (GVHD) risk and lower chance of cancer formation as compared to embryonic stem cells make adult stem cells as a promising therapeutic and treatment candidate as well as a potential research subject (Leeb *et al.,* 2011). An effective adult stem cells isolation method would provide a better chance to study the stem cells efficiently. Size of stem cells should be taken into consideration in peripheral blood stem cell isolation procedure since it may enrich the isolation product when combined with other technologies such as nanotechnology. Previous studies showed distinguishably larger hematopoietic stem cell (HSC) sizes can be applied in fabricating microfluidic devices that worked on bone marrow sample to isolate HSC and the result shows up to 98% enrichment (Schirhagl *et al.,* 2011).

Peripheral blood suspension stem cells used in this research were adult stem cells with multi-potential ability (Ruzanna *et al.,* 2011). Adult stem cells are a part of developmental continuum in tissues such as blood since the cells yet to differentiate and co-exist with the differentiated mature cells in those tissues. Besides, adult stem cells are defined by their capacity for unlimited self renewal during the lifetime of the organism and multi-potential differentiation ability to differentiate into several lineages through symmetrical and asymmetrical division (Hall *et al.,* 1989; Daniella *et al.,* 2012). Peripheral blood is a potential stem cell source since the isolation method is non-invasive and the source is easily available compared to bone marrow. Previous studies proved that MNCs isolated from peripheral blood consist of adult stem cells, i.e., hematopoietic stem cells and mesenchymal stem cells (Yazid *et al.,* 2011).

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KIT gene (CD117) is a stem cell factor receptor that known to trigger some important biological effects when bound by their ligand, stem cell factor (SCF). In hematopoietic stem cells (HSC), the binding of SCF to KIT promote the survival, proliferation, and differentiation of HSC (Broudy, 1997; Nakamura, 2004). Signaling lymphocytic activation molecule family member 1 (SLAMF1) also known as CD150 is a cell surface receptor that plays an important role in proliferation and self renewal of HSC (Kent *et al.,* 2009). In this research, both *KIT* and *SLAMF1* genes were used as biomolecular marker for stem cell. Study on the relationship between cell size and proliferation potential of stem cell population from three different culturing terms will enhance our knowledge on the possibility of isolating certain culturing term stem cell population based on their population sizes and proliferation ability in complete culture media.

MATERIALS AND METHODS

Isolation and culturing of peripheral blood mononucleated cells

The human peripheral blood sample was obtained from three healthy donors with the assistance of Universiti Kebangsaan Malaysia (UKM) medical centre staff. Peripheral blood sample was diluted three times with Hank's Balanced Salt Solution (HBSS). The mononucleated cells (MNCs) were isolated from diluted blood sample through density gradient centrifugation using Ficoll Paque™ Plus. Blood sample was layered onto Ficoll Paque™ Plus at ratio 1:1.5 and centrifuged at 400 g for 20 minutes at room temperature. The layer containing MNCs was carefully pipetted into a new 15 mL Falcon tube and washed down using Hank's Balanced Salt Solution (HBSS) and Phosphate Buffered Saline (PBS). The MNCs were cultured in 6 well plates with 2 mL of complete media containing α-Minimal Essential Medium (α-MEM) with 10% (w/v) Newborn Calf serum (NBCS) and 2% (v/v) penicillin/streptomycin antibiotic solution. The cultured plate was stored at 37ºC in humidified atmosphere with 5% CO_2 for 30 days with medium exchange every 3 days.

Proliferation Analysis

Proliferation analysis was carried out by counting the viable cells using haemacytometer. Proliferation analysis usually started with 1 x 10^5 cells/mL seeded in 24-well plate. Firstly, the total number of cells in the 2 mL culture media was counted on the day of isolation (day 0). After 4 days, 1 x 10^5 cells/mL were seeded in 24-well plate with 1 mL of complete media containing α-MEM with 10% (w/v) NBCS and 2% (v/v) penicillin/streptomycin. Every time before 10 µL of cell sample was taken out for cell counting, the medium containing MNCs were mixed evenly. The sample was stained with 10 µL Trypan Blue 0.4% (v/v) staining at ratio 1:1 and loaded on haemacytometer. Viable cells were counted under microscope and the total number of viable cells in the sample media was calculated. As stated above, fixed amount of cells were seeded in 24-well plate with 1 mL of complete media. Then, cell counting continued for each day until 30 days. The seeded cells in 1 mL complete media were subcultured when the number of calculated cells exceeded the fixed number of cells. The culture media was exchanged every 3 days.

RT-PCR Analysis

Total RNA was extracted from peripheral blood MNCs using Tri-Reagent® kit (Sigma, USA) by following manufacturer's instruction. RT-PCR amplification carried out on 1 microgram total RNA by using an Access RT-PCR System kit (Promega, USA). Reverse transcription was carried out at 45ºC for 45 min to synthesis the first-strand complementary DNA (cDNA) and followed by Avian Myeloblastosis Virus reverse transcriptase inactivation at 94ºC for 2 min. Second strand cDNA synthesis and PCR amplification consisted of 40 cycles of denaturation at 94ºC for 30 sec, primer annealing at 53ºC (*KIT*) and 54ºC (*SLAMF1*) for 1 min, extension at 68°C for 2 min, and final cycle at 68°C for 7 min. Specific primer sequences were as stated in Table 1. The RT-PCR products were analyzed through 1% (w/v) agarose gel electrophoresis and then sequenced.

Table 1. Primer Sequences Used in RT-PCR

Gene	Primer	Sequence
KIT	Sense	5'TCCTTGACCTTCGTGCTGT3'
	Antisense	5'CCTTCCTGCTTTCCCGTAT3'
SLAMF1	Sense	5'CTCTGCGTTCTGCTCCTAC3'
	Antisense	5'TTGGTCACTTCTGGGTCTG3'

Size Measurement

MNCs size measured starting from day 0, 3, 7, 10, 14, 17, 21, 24, 27, and 30. About 1 x 10^5 cells/mL MNCs from culture media were washed with PBS at 400 g for 5 min at room temperature. Then, the MNCs were mounted on glass slide through cytocentrifugation at 1000 g for 5 min at room temperature. The MNCs monolayer on glass slide was air dried for 15-20 min before subjected for methanol fixation and staining procedures. The mounted cells were fixed on glass slide via covering them with methanol for 1 min. Then, the methanol was poured off from glass slide. May Grunwald-Giemsa staining solution was used for staining of the fixed cells. Firstly, the MNCs on glass slide were covered with May Grunwald solution for 15 min and then removed via poured off from glass slide. Then, the cells was stained using Giemsa staining solution for 10 min. Excess staining on glass slide was washed with deionized distilled water and left for air dried. Attached camera in Olympus Microscope was used to take pictures of MNCs monolayer. About 50 MNCs were randomly selected for size measurement using "CELL B" software in Olympus Microscope.

Statistical Analysis

Data analyses were carried out using paired t-test, ANOVA, and correlation from Minitab 14 software. The value of $p<0.05$ showed significant differences between tested data.

RESULTS AND DISCUSSION

Proliferation Rate and Cell Size of Peripheral Blood Suspension Mononucleated Cells

Analysis of cell viability helps to determine the proliferation potential of suspension mononucleated cells (MNCs). Figure 1 showed the result of cell viability of suspension MNCs for 30 days. The isolated MNCs were left in complete culture media for 4 days so that a more homogenous population of suspension MNCs can be obtained. Then, the suspension MNCs from 4 days old *in vitro* culture were fixed at 1 x 10^5 cells/mL in 24 well plate and considered as day 0 for proliferation analysis. Paired t-test analysis showed a significant ($p<0.05$) difference in number of MNCs between day 7, 14, and 30 as compared to day 0. There was an increase of cell number from day 0 to day 30 of the culturing period as shown by growth curve in Figure 1. Population doubling time (PDT) represent the time taken by a population of cells to double their starting number of cells. Higher PDT value means lower proliferation rate. Paired t-test for PDT and increase in the number of cells showed significant difference between day 7, 14, and 30 as compared to day 0 (Table 2). MNCs from long term culturing population revealed the lowest PDT value and greatest increase in number of cells, showing that long term cultured MNCs have the highest proliferation rate as compared to the other two culturing terms. The ability of MNCs to proliferate

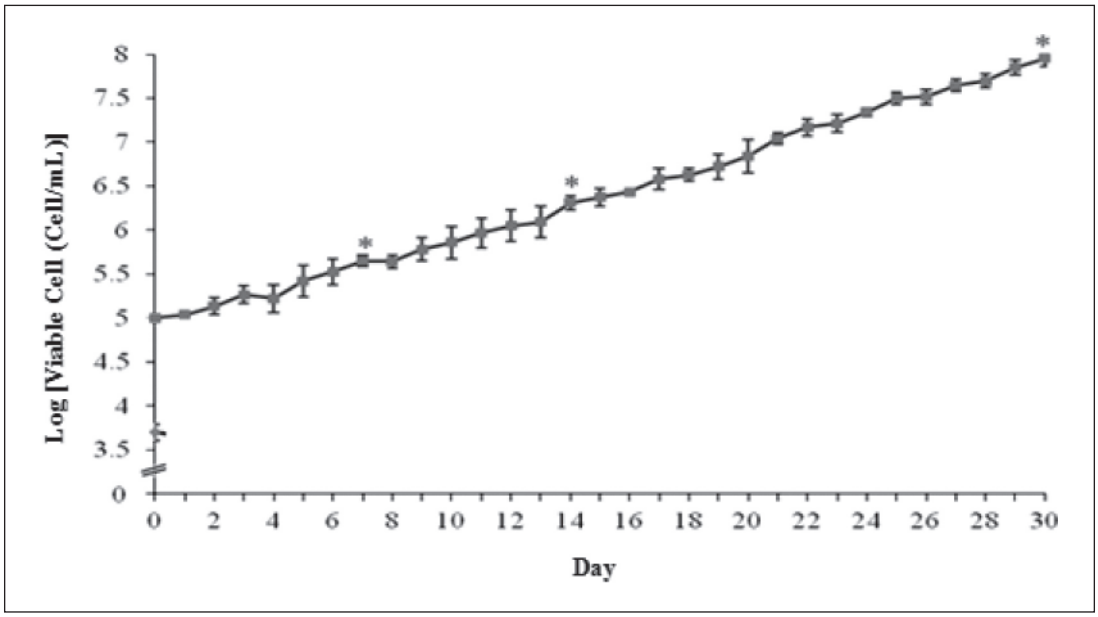

Fig. 1. Growth Curve of Human Peripheral Blood Stem Cells for 30 days.

Table 2. Size and Proliferation Rate of Human Peripheral Blood Stem Cells from Three Different Culturing Terms

Culturing Term	Day	Size (μM)	Population Doubling Time, PDT (Hour)	Increase in Cell Number
Short	7	7.00±0.7[a]	77.5	4.5±0.6
Medium	14	6.36±0.8[b]	38.5	20.7±3.9
Long	30	6.28±0.8[c]	17.1	913.3±32.2

Values are expressed as means ± SD. Values are not sharing superscripts letters (a, b, c) differ significantly at $p<0.05$.

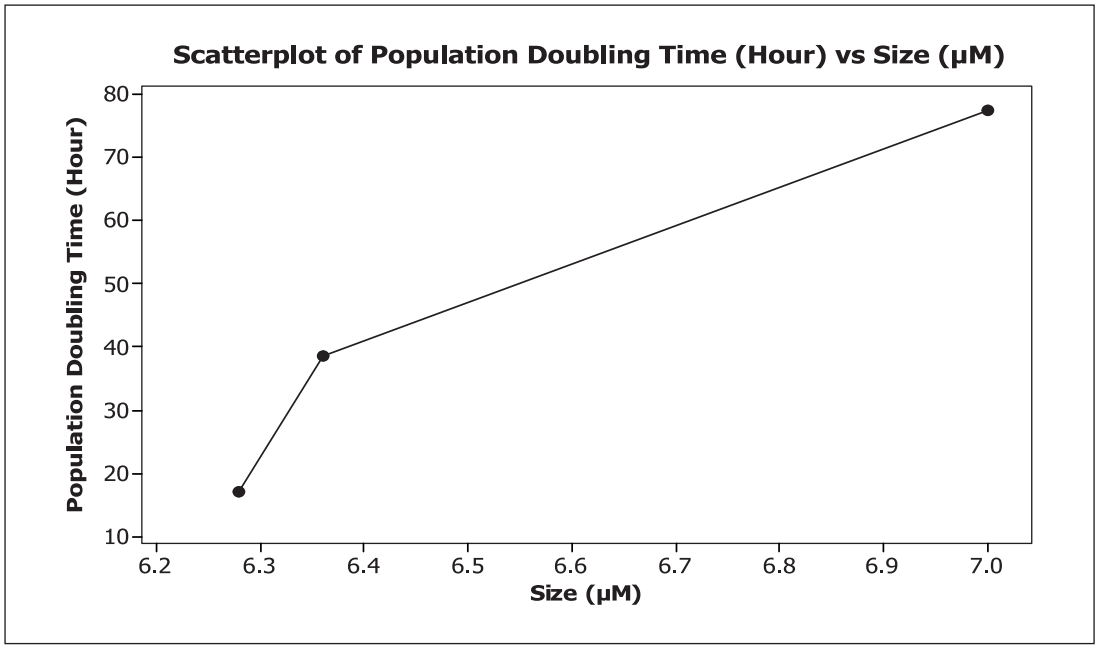

Fig. 2. Correlation Analysis between PDT and Cell Size of Human Peripheral Blood Stem Cells.

well in complete media until 30 days also indicated that the complete culture media are suitable for long term culturing population. The size of MNCs for each culturing term population in Table 2 were tested using ANOVA in Minitab 14 and the result showed significant ($p<0.05$) difference among each others. Figure 2 showed the result for correlation analysis of population PDT versus cell size i.e. the PDT value increases as the cell size increases. Since high PDT value indicates low proliferation rate, the results showed that an increase in the cell size reduces the proliferation rate of the MNCs.

Suspension Mononucleated Cells in each Culturing Term Population are Suspension Stem Cells

The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis was carried out by using equal amount of RNA i.e., 80 ng/μL. The expected band size for *KIT* and *SLAMF1* genes are 316bp and 403bp, respectively. Presence of each expected band size in electrophoresis gel profile A and B in Figure 3 gave a positive result for the presence of both genes in the RT-PCR product. Besides, an increase in the band intensity for both genes was found from day 7 to day 30. The increase in band intensity may be due to the increase in the number of suspension stem cells in population approaching more homogenous population with extension in culturing period. The presence of stem cell markers during RT-PCR analysis concluded that MNCs in each culturing term population can be claimed as stem cells.

CONCLUSION

Analyses of proliferation rate and cell size showed short term stem cells having the largest cell size with the lowest proliferation potential. Meanwhile, medium term stem cells gave rise to smaller stem

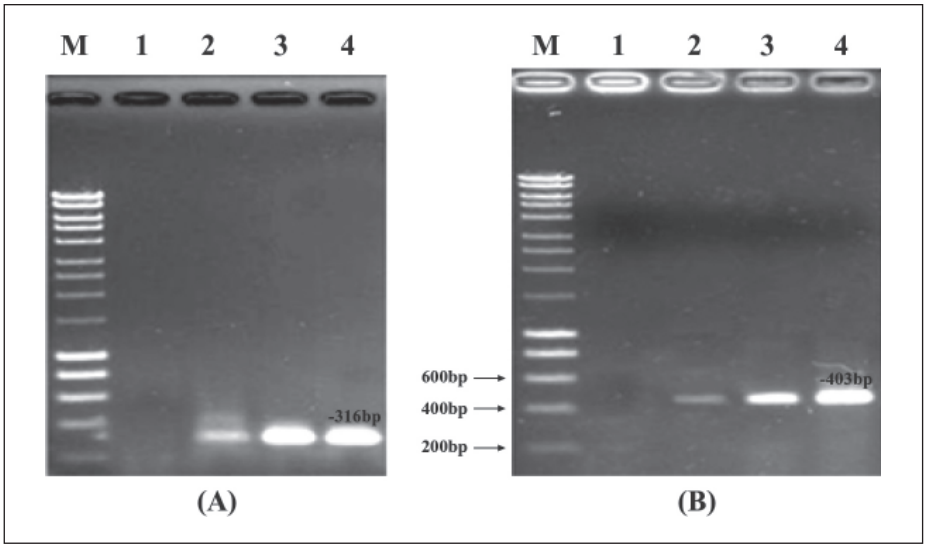

Fig. 3. Electrophoresis Gel Profile (1%) (w/v) of Human Peripheral Blood Stem Cells from Three Different Culturing Terms for RT-PCR Product (A*) KIT* gene and (B) *SLAMF1* gene. Lane M, 1, 2, 3, and 4 represent Hyperladder 1 Marker, Day 0, Day 7, Day 14, and Day 30, respectively.

cell population with higher proliferation potential as compared to short term stem cells. Long term stem cells were shown to have smallest cell size with the highest proliferation potential. Each culturing term cell population has their own sizes and can be isolated based on those sizes.

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REFERENCES

Broudy, V.C. Stem cell factor and hematopoiesis. 1997. *Blood,* **90:** 1345-1364.

Daniela, G.H., Sally, A.A. & David, J.A. 2012. Stem cells and veterinary medicine: Tools to understand diseases and enable tissue regeneration and drug discovery. *The Veterinary Journal,* **191:** 19-27.

Hall, P.A. & Watt, F.M. 1989. Stem cells: the generation and maintenance of cellular diversity. *Development,* **106:** 619-633.

Kent, D.G., Copley, M.R., Benz, C., Wo¨hrer, S., Dykstra, B.J., Ma, E., Cheyne, J., Zhao, Y.J., Bowie, M.B., Zhao, Y., Gasparetto, M., Delaney, A., Smith, C., Marra, M. & Eaves, C.J. 2009. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood,* **113:** 6342-6350.

Leeb, C., Jurga, M., McGuckin, C., Forraz, N., Thallinger, C., Moriggl, R. & Kenner, L. 2011. New perspectives in stem cell research: beyond embryonic stem cells. *Cell Proliferation,* **44:** 9-14.

Nakamura, Y., Tajima, F., Ishiga, K., Yamazaki, H., Oshimura, M., Shiot, G. & Murawakia, Y. 2004. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Experimental Hematology,* **32:** 390-396.

Ruzanna, A.K., Ariffin, S., Wahab, R., Senafi, S. & Fahrul, Z.H. 2011. Differentiation potential of human suspension mononucleated peripheral blood cells. *African Journal of Biotechnology,* **10(52):** 10756-10764.

Schirhagl, R., Fuereder, I., Hall, E.W., Medeiros, B.C. & Zare, R.N. 2011. Microfluidic purification and analysis of hematopoietic stem cells from bone marrow. *Lab Chip 11*: 3130-3135. DOI: 10.1039/c1lc20353c.

Yazid, M., Ariffin, S., Senafi, S. & Wahab, R. 2011. Determination of Mononucleated Cells Heterogeneity: A Preliminary Molecular Approach. *Proc. of the Annual International Conference Meeting on Stem Cell Research (SCR 2011)*. Pg. 13-16.